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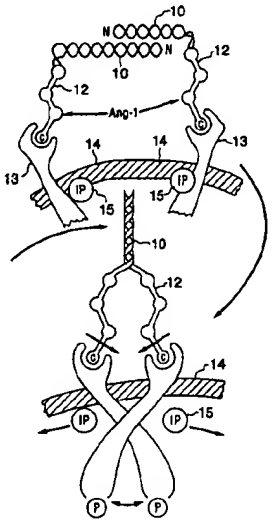

(57) Abstract: Angiopoietin analogs capable of binding to and activating TIE2 receptors by dimerization thereof, the angiopoietin analogs being proteinaceous molecules having at least 2 angiopoietin-like TIE2 binding domains.

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ANGIOPOIETIN ANALOGS

FIELD OF THE INVENTION

This invention relates to medical treatments, and compounds and compositions useful therein. More particularly, it relates to novel angiopoietin analogs, and therapeutic and medical uses thereof.

BACKGROUND OF THE INVENTION

Angiopoietins, Ang, are secreted proteins (angiogenic, growth-like factors) which act as ligands for the receptor tyrosine kinase TIE2 (also known as TEK) which are expressed almost exclusively on the membranes of endothelial cells and early hematopoietic cells. The TIE receptor family is critically involved in vasculogenesis (the formation of blood vessels de novo during embryonic development, and angiogenesis (the formation of new vessels by sprouting from pre-existing vessels). Angiogenesis accompanies and is required for the normal growth of post-natal tissues, for the continuous remodeling of the adult female reproductive system, and in pathological situations such as wound healing and tumor growth.[1]

Two different but similar angiopoietins have been identified and reported to date, Ang1 and Ang2. Suri and co-workers[1] reported that Ang1 is a primary physiologic ligand for TIE2 and has a critical *in vivo* action during vasculogenesis. Ang1 both binds to and activates TIE2, whereas Ang2 binds to TIE2 but does not activate it. Rather, Ang2 can inhibit phosphorylation of the TIE receptor induced by Ang1.

TIE2 receptors, in common with other tyrosine kinases, become activated by dimerization (chemical linking) or oligomerization (clustering) of such receptors located in close proximity to each other on the cell surface. Such dimerization results in tyrosine phosphorylation (i.e. adding a phosphate moiety to a tyrosine residue of the cytoplasmic tail of the receptor). Thus, it appears that

Ang1 can bind the TIE2 and produce dimerization of the ligand receptor complex leading to autophosphorylation and activation of TIE2. In contrast, although Ang2 binds TIE 2 equally well, it does not appear to be able to induce receptor phosphorylation, possibly by being unable to aggregate this receptor complex.

Ang1 accordingly has potential medical utility in treatment of patients, including human patients, having conditions requiring accelerated or increased vasculogenesis or angiogenesis (e.g. wound victims). Therefore, activation of TIE-2 is dependent on its dimerization (or oligomerization) and the agiopoietins demonstrate variable abilities to to activate TIE-2, possibly related to variable abilities to promote association of the receptor-ligand complexes. Thus, it may be beneficial to generate novel angiopoietin-like molecules which have greater ability to aggregate TIE-2 receptors, and thus will exhibit greater biological activity or be less likely to be antagonized.

Accordingly, it is an object of the present invention to provide novel compounds and compositions having the effect of activating the TIE2 receptor in mammalian cell membranes.

The amino acid sequence for Ang1 has been elucidated and published.[2] Not very much is known, however, about the structure of the Ang1. It appears to have at least two domains, one of which is generally of coiled confirmation, (the "coiled-coil domain") and the other of which, constituting about half the natural molecule, exhibits a substantial degree of homology with fibrinogen (the "fibrinogen-like domain"). It has recently been reported that binding to TIE2 takes place at the fibrinogen-like domain of Ang1, rather than at the coiled-coil domain.

SUMMARY OF THE INVENTION

According to the present invention, there are provided angiopoietin analogs capable of binding to and activating TIE2 receptors by dimerization

thereof, the angiopoietin analogs being proteinaceous molecules having at least two angiopoietin-like TIE2 binding domains. Such analogs of angiopoietin bind to two separate but closely related TIE2 receptors on an endothelial cell membrane, and bring them together, so as to activate the receptors by phosphorylation and to promote the process of angiogenesis.

By "angiopoietin-like" (Ang-like) domains, as the term is used herein, is meant domains of the molecule which exhibit sufficient homology to the corresponding domain of natural angiopoietin as to have substantially similar properties and activities to natural angiopoietin. These may be polypeptides having substantial homology of amino acid sequence to the natural angiopoietin, for example at least 50% identity of amino acid sequence, more preferably at least 80% and most preferably at least 95% identity of amino acid sequence with the natural product. Ang-like domains may include polypeptides having significant homology to one or both of the fibrinogen-like domain and the the coiled-coil domain of native Ang-1, to have substantially similar properties and activities to natural angiopoietin, despite having other regions of amino acids lacking significant homology to native Ang-1. Preferably such polypeptides have at least 80% homology of amino acids to the naitve Ang-1 fibrinogen-like domain or the coiled-coli domain. More preferably such polypeptides have at least 95% homology to one of these regions. It will be understood by those skilled in the art that various conservative amino acid substitutions which will not significantly alter the function of the protein are possible and contemplated.

As used herein, the term "analogous" refers to a polypeptide having a tertiary structure sufficiently similar to that of the native polypeptide to permit the "analogous" polypeptide to perform substantially the same function as the native polypeptide.

Unrelated molecules or portions thereof that have similar properties and functions, e.g. anti-TIE2 antibodies that could activate the receptor by promoting TIE-2 association, are also within the term. Such molecules can be

readily identified, in the light of the disclosure herein, through molecular modeling methods known in the art and standard screening assays.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted, it has been reported that the fibrinogen-like domain constitutes or includes the TIE2 binding site of Ang1. Accordingly, a preferred embodiment of the invention is an Ang-like chimera containing a proteinaceous molecule having at least two fibrinogen-like domains. However, the invention also embraces an Ang analog comprising a proteinaceous molecule having at least two coiled-coil domains, for other utilities involving interaction with the TIE2 binding site of Ang1.

In another embodiment of the invention, there is provided an Ang-like chimera having fibrinogen-like domains including glycosylation similar to the glycosylation of fibrinogen-like domains of native Ang-1. Preferably the fibrinogen-like domains of the Ang-like chimera are glycosylated on at least 50% of the typical glycosylation sites of native Ang-1. It is also preferable that glycosylation of Ang-like chimeras involve sugar moieties of a similar size and reactivity to those involved in the glycosylation of native Ang-1.

It is preferred to include linker or spacer sequences between the Ang-like TIE2 binding domains of Ang (chimeras) analogs of the present invention. Such sequences can be tailored to have appropriate length to accommodate the spacing between and geometry of the TIE2 receptors on the cell membrane, and appropriate flexibility for cooperation therewith. Such arrangements lead to more efficient binding and enhanced activation of the receptors. Appropriate linker or spacer sequences may be one or more angiopoietin-like domains which are not active in TIE-2 binding such as the coiled-coil domain. Accordingly, one preferred product according to the present invention is an Ang analog which is a proteinaceous molecule having at least two Ang-like fibrinogen-like domains and at least one Ang-like coiled-coil domain disposed therebetween. Another specific

product is an Ang analog which is a proteinaceous molecule having at least two Ang-like coiled-coil domains and at least one Ang-like fibrinogen-like domain disposed therebetween. Such products, being largely analogous to the native Ang1 of known amino acid sequence, are the simplest to make by standard genetic engineering techniques. Another embodiment of the invention comprises Ang-like proteinaceous molecules having multiple duplications of the fibrinogen-like domain, such that one chimeric molecule has the ability to bind three or more TIE-2 receptors. Genes and nucleic acid constructs capable of directing the expression of proteinaceous molecules having at least two angiopoietin-like TIE2 binding domains, especially fibrinogen domains as described above, constitute a further aspect of the present invention.

Another preferred embodiment of the invention is proteinaceous molecules which include, in addition to an Ang-like domain capable of binding to TIE2, at least one vascular endothelial growth factor (VEGF)-like domain capable of binding to VEGF receptor, VEGFR1 and/or VEGFR2 KDR on the endothelial cells. Such a molecule may effect heterodimerization of TIE2 to VEGF-specific receptors, for additional, powerful angiogenic effects. A still further embodiment comprises molecules having one or more Ang-like binding domains, or VEGF receptor-binding domains which have been engineered to efficiently produce self-association (for example incorporating a "leucine zipper"). This will effectively result in clustering of TIE-2 or VEGF receptors (or heterodimers thereof) together on the cell membrane and thus potentiate the angiogenic response.

There is evidence that Ang-1 analogues according to preferred embodiments of the present invention not only dimerize TIE2 receptors, but may in fact proceed beyond dimerization thereof to cluster or otherwise associate 3 or even more of the TIE2 receptors, perhaps thereby creating greater activity in the TIE2 receptors. Dimerization is of course involved and included, whenever two or more TIE2 receptors are bound together through a common binding entity, so as to effect phosphorylation of the receptors.

As disclosed by Davis et al.[2], angiopoietin 1 is a glycoprotein of molecular weight approximately 70 kDa having 498 amino acids, and carrying a number of sugar residues. The region consisting of residues 100-280 constitutes the coiled-coil domain of Ang1. The region consisting of residues 280-498 has strong similarity to a family of proteins including fibrinogen, and constitutes the fibrinogen domain of Ang1.

BRIEF REFERENCE TO THE DRAWINGS

Figure 1 of the accompanying drawings is a diagrammatic illustration of a proposed mechanism for the activation of TIE2 by native Ang1;

Figures 2-7 are diagrammatic illustrations of various preferred embodiments of the present invention;

Figure 8 is a diagrammatic presentation of the vector constructs made and used in Example 2 below.

SPECIFIC DESCRIPTION OF THE MOST PREFERRED EMBODIMENTS

With reference to Figure 1, native Ang1 has a coiled coil domain 10 and a fibrinogen domain 12. The C-terminal region of each of two Ang1 molecules, on the fibrinogen domain 12, is involved in binding to a respective, adjacently positioned TIE-2 receptor 13 on an endothelial cell membrane 14, in the vicinity of associated and as yet unidentified regulatory protein molecules 15. Upon binding to the TIE-2 receptors 13, the coiled-coil domains 10 of the two Ang1 molecules are brought together, thereby dimerizing the TIE2 receptors 13, with elimination of the putative regulatory protein molecules 15. This dimerization activates the TIE2 receptor, by tyrosine autophosphorylation, indicated by "P" in Fig. 1. This mechanism is put forward as a proposal for the mechanism for activation of TIE2 by native Ang1, for better understanding of the present invention, but is not to be construed as a limitation to any particular theory of

action.

In each of the accompanying Figures, the Ang1 analogs according to the present invention are shown to have a coiled-coil domain 10 and one or more fibrinogen-like domains 12, either accompanied by a coiled-coil domain 10 as spacer, or by an alternative, biochemically acceptable spacer, or alternatively incorporating other angiogenesis or vasculogenesis mediating binding domains such as VEGF.

The proteinaceous molecule diagrammatically illustrated in Figure 2 comprises two fibrinogen domains 12, spaced by a coiled-coil domain 10. Both fibrinogen domains 12 are available for binding to TIE2 receptors on endothelial cell membranes, to effect dimerization and hence activation thereof in angiogenesis. Both fibrinogen domains 12 can be identical in sequence to the fibrinogen domains of natural Ang1. Similarly, the coiled-coil domain 10 can be analogous to the natural coil domain of Ang1 or a portion thereof, depending on the length required for appropriate TIE2 dimerization.

Figure 3 is effectively the reverse situation to that of Figure 2, and is useful in other possible interactions with the TIE2 binding site. The coiled-coil domains of the embodiment of Figure 3 can be analogous to the coiled-coil domain of natural Ang1, and the fibrinogen domain 12 can be analogous to fibrinogen domain of natural Ang1, of appropriately tailored length as described above. Such an Ang1 analog is preparable by genetic engineering techniques, since the sequences are known.

In Figure 4 of the accompanying drawings, there is illustrated an embodiment of the invention comprising a proteinaceous molecule having two domains 12 analogous to the naturally occurring fibrinogen domain of Ang1, and a synthetic linker sequence 17 of biochemically acceptable chemical constitution and acting as a synthetic spacer for the TIE2 receptor binding domains 12. The linker 17 is of length and flexibility suitable to promote TIE2 binding and

dimerization. Dextran, hydroxyethyl starch and polyethylene glycol may be suitable choice of chemical constitution for group 17.

Figure 5 of the accompanying drawings, diagrammatically illustrates another preferred embodiment of the present invention, namely a proteinaceous molecule having a TIE2 binding domain 12 which is an amino acid sequence analogous to the fibrinogen domain of Ang1, and a second domain 16 which is analogous to the tyrosine kinase receptor binding domain of vascular endothelial growth factor VEGF. It is known that VEGF plays an important role in angiogenesis, by binding and activating members of its family of tyrosine kinase receptors, such as Flt-1, Flt-4 and Flk-1/kdr. Accordingly, the proteinaceous molecule diagrammatically illustrated in Figure 5 will effect heterodimerization of tyrosine kinase receptors on the membrane of endothelial cells, to cause angiogenesis effects. If desired, an inert, biochemically acceptable spacer sequence can be provided between the Ang1 receptor binding sequence 12 and the VEGF receptor binding sequence 16 of Figure 5, such as a sequence 17 of Figure 4, to provide appropriate spacing and flexibility of the molecule for enhanced binding.

Figure 6 of the accompanying drawings shows a further preferred embodiment, comprising two half-molecules in which the proximal Ang1 coiled-coil domain ends are replaced by leucine zippers for similar dimerization and motifs 18,20 which effect binding of the molecule together. Each half-molecule has a TIE2 binding domain 12, attached to the other end of the coiled-coil domain 10. Figure 7 shows a similar, alternative embodiment having two terminal TIE2 binding domains 12 and two terminal KDR binding domains 22, engineered with oligomerization motifs to promote tetramerization of modified TIE2 and KDR binding proteins. Coiled coil sequences 10 could also be replaced by inert polymer sequences 17.

The preparation of the proteinaceous molecules of the present invention can be accomplished, from a knowledge of the Ang1 amino acid

sequence already published, by standard amino acid coupling techniques, e.g. using solid state supports to which the initial amino acid is chemically bound, followed by sequential addition of the individual amino acids to grow the protein chain step-by-step in attachment to the solid state substrate, protectant groups being used where appropriate to predetermine the course of reaction (Merrifield synthesis). In the case of embodiments of the invention such as that shown in Figures 2, 3, and 5, where the total molecule is proteinaceous, the entire molecule can be synthesised in this manner. In the case of embodiments such as those shown in Figures 4, 6 and 7, the proteinaceous portions of the molecules 12, 16 can be synthesized in this manner, and then chemically attached to the spacer group 17 by known methods, depending on the choice of identity of spacer group 17.

It is preferred, however, to make the proteinaceous molecules of the present invention, or at least the proteinaceous portions of them, by genetic engineering techniques. The sequences of the proteinaceous portions 10, 12, 16 are known and published, so that nucleic acid sequences i.e. genes coded for their expression can be synthesized. Such nucleic acid sequences can be incorporated into plasmids or the like, and introduced into host cells where they will express the required amino acid sequences upon culturing and growth of the cells. Preferably, host cells are selected which carry out glycosylation of the amino acid sequences similar to natural glycosylation of native Ang-1. Techniques for obtaining amino acid sequences by such genetic engineering methods are well known and well established in the art, and do not require the exercise of more than ordinary skill in the art.

Whilst it is possible and within the scope of the present invention to produce the wholly proteinaceous molecules such as those diagrammatically illustrated in Figures 2, 3 and 5 in a single step of expression from a synthetic gene coding for the entire sequence, it may be preferred to prepare such molecules as individual component sequences, and then to link them together as desired. By such linking techniques, one can more easily prepare proteinaceous molecules

analogous to the natural products Ang1. For example, in the case of the embodiment diagrammatically illustrated in Figure 2, its production in a single stage from a gene coding for the entire amino acid sequence 12-10-12, will result in a product having a free N-terminus at the end of one sequence 12 and a free C-terminus at the end of the other sequence 12. This is because the expression of the amino acids based upon the protein sequence takes place in the N-C direction. In the natural product Ang1, however, the free ends of the presumed TIE2 binding domains are constituted by a C-terminus. This might compromise binding of the second fibrinogen-like domain to TIE2 if a free N-terminus were required in this interaction. One can prepare products which more closely resemble the natural Ang1 products, by a process in which each recombinant molecule would have only one TIE2-binding domain, e.g. by gene expression, and then individual Ang1 analogues are subsequently linked together in a manner so that there is a free C-terminus on each TIE-2 binding domain 12.

Appropriate methods of linking together amino acid sequences after they have been prepared, e.g. to assembly a proteinaceous molecule as illustrated in Figure 2 or Figure 3 are known in the art. The preferred such method is by use of "leucine zippers", in which amino acid sequences to be joined together each terminate in a sequence of leucine units e.g. 6-10 leucine units long. Such leucine terminated amino acid sequences will bind together, through chemical interaction of the leucine chains. Accordingly, preparation of "half molecules" with leucine zippers can be undertaken, a relatively simple synthesis, and the half molecules will associate into full molecules as illustrated, either extermely or internally of the patient, i.e. after administration.

Administration of the proteinaceous molecules, angiopoietin analogs, of the present invention, to patients for purposes of enhancing angiogenesis, can be by standard therapeutic administration techniques such as injectable solutions, but is preferably effected by means of gene therapy. Accordingly, another aspect of the present invention comprises genetic elements containing gene sequences capable of *in vivo* expression of angiopoietin analogs as described or defined

herein, for administration to patients in need thereof. Such genetic elements include recombinant viruses containing the desired DNA sequences, plasmids and cells previously transfected with such viruses or plasmids.

The construction and administration of such genetic elements, for use in gene therapy, is within the skill of the art.

The invention is further described, for illustrative purposes, in the following specific experimental reports, constituting the current best mode of conducting the invention.

MATERIALS AND METHODS

Cell lines and culture:

Human umbilical vein endothelial cells (HUVEC), rat aortic A10 smooth muscle cell (A10SMC) and human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The EA.hy926 endothelial cell line was a gift from Cora Edgell, University of North Carolina. HUVEC were maintained in culture in Ham's 12 medium, supplemented with 15% fetal bovine serum (FBS), penicillin (500 U/ml), streptomycin (50µg/ml) and heparin (100µg/ml), (all from Gibco/BRL, Burlington, ON, Canada), and endothelial cell growth factor (ECFG 20µg/ml, Boehringer Manheim, Laval, PQ, Canada) and equilibrated with 95% air and 5% CO_2 at 37°C. Cells were used between passages 13 and 18 in these experiments A10 SMC and HEK293T cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, as described above, in the presence of x HAT (Gibco/BRL).

Example 1-Generation and use of Ang1 and Ang2 expressing cells:

Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2) cDNAs were subcloned into the mammalian expression vector pSecTagB/Myc-His(+)

(Invitrogen, Mississauga, ON) to generate Myc-Histidine epitope-tagged constructs (Ang1MH and Ang2MH). These epitope-tagged constructs are prepared for ease of purification and identification, since antibodies to Ang, although available, are not wholly reliable or satisfactory use, whereas antibodies to Myc are available and are more reliable in practice. The histidine tag allows for purification of these compounds using a copper column. A portion of Ang1 lacking the leader signal sequence was cloned into vector SignalpIg-plus (Novogen, Madison, WI), to generate vector Ang1Fc as previously reported (see Teichert-Kuliszewska K. et al, "Cardiovascular Research" 2000 (in press)). To produce stable cell lines expressing Ang1MH and Ang2MH, HEK293T and A10 SMC were transfected with 10μg of pSecTagB-Ang1MH or with pSecTagB-Ang2MH plasmids. Transfections of HEK293T cells were performed using Lipofectin reagent and OPTI-MEM medium (both from Gibco/BRL) according to the manufacturer's instruction. A10 SMC were transfected using SuperFect Transfection reagent (Qiagen, Mississauga, ON) according to the manufacturer's recommendation. Transfectants were selected in 1mg/ml zeocin (Invitrogen) and individual colonies isolated by ring cloning, pooled and expanded. To produce Ang1Fc expressing stable cell lines, HEK293T cells were transfected with 10μg of SignalpIg-plus-Ang1 using Lipofectin reagent. Transfectants were selected in 1.5 mg/ml G418 (Gibco/BRL) and individual colonies were pooled and expanded as above. Conditioned medium (CM) was collected during 24 hours incubation of the stable transfected cells in DMEM supplemented in 0.1% or 10% FBS. Ligand production was confirmed by RT-PCR and immunoprecipitation with c-Myc antibody (Invitrogen), following Western analysis. HUVEC were treated for 40 hrs with Cm from either mock-transfected. Ang1MH, Ang1Fc, Ang1FC-depleted or Ang2MH. To deplete Ang1Fc from HEK293T CM, 10 ml of media was incubated with 500μl 20% protein A-Sepharose beads that had been coated with Tie2-Fc rocked for 12 hours. The beads were spun down and the remaining supernatant was applied to HUVEC Human Ang1*, Ang2 and soluble Tie2-(s Tie2-Fc) were obtained from Regeneron Pharmaceuticals, Inc., Tarrytown, NY. Ang1* and Ang2 recombinant proteins were produced as described previously. Ang1* is a genetically engineered variant that retains properties similar to wild type Ang1. In Ang1*, the non-conserved cysteine

at residue 245 has been mutated to the corresponding serine residue of Ang1, and the first 77 aminoacids of human Ang1 have been replaced with the first 73 residues of Ang2. The recombinant proteins were prepared in buffer containing 0.05 mol/L Tris-Hc1 pH 7.5, 150 mmol/L NaCl and 0.05% Chaps. Soluble Tie2-Fc is a recombinant fusion protein consisting of the ectodomain of the Tie2 receptor fused to the Fc portion of human IgG, which as constructed, produced and purified as described. Ang1* and Ang2 were used at concentrations from 0-2000 ng/ml for 24-48 hrs.

Detection of angiopoietins in conditioned media:

Conditioned media was clarified by centrifugation prior to immunoprecipitation. Ang1MH and Ang2MH were precipitated from 10 ml of CM with 2μg of purified rabbit anti-mouse IgG and recovered using protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). The precipitated proteins were eluted in twice-concentrated sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min, separated using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane (Novex, Helixx Technologies, Scarborough, ON) using a semi-dry transfer apparatus (BioRad), according to manufacturer's instructions. Filters were blocked in 5% non-fat milk in TBST buffer (10 mmol/L tris (pH 7.5), 150 mmol/L NaCl and 0.1% Tweeen20) prior to immunoblotting with anti-Myc antibodies at 1:5000 dilutions according to the supplier's instruction (Invitrogen). An HRP conjugated anti-mouse antibody coupled with ECL reagent (Amersham) was used to visualize angiopoietin-specific band (70 kDa). Ang1Fc was detected with HRP-conjugated donkey anti-human IgG H+L (Jackson ImmunoResearch, West Grove, PA) as 97 kDa protein.

Tie2 autophorsphorylation:

For Ang1 and Ang2 induced stimulation of EA.hy926, (an endothelial cell line used previously to study Tie2 activation), cells were serum-starved for 6 hours, pretreated with 1 mmol/L sodium orthovanadate (phosphatase inhibitor), pH

8, for 10 min at 37°C, and stimulated with 5ml of conditioned medium in the presence of sodium orthovanadate for 10 min. Alternatively HUVEC were maintained overnight in McDB 131 medium (Gibco/BRL) with 1% FBS, and treated with Ang1* or Ang2 pure proteins (30 ng/ml each) for 5 min, 1 or 24 hours. In some experiments, cells were pre-treated for 24 hours in the presence of factors, medium was removed, cells were then serum-starved in MCDB 131 medium for 2 hours, followed by 5 min rechallenge with Ang1* or Ang2. After the stimulation of cells were solubilized with RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mmol/L Tris, pH 7.6, 50 mmol/L sodium fluoride, 150 mmol/L sodium chloride 1 mmol/L EDTA, 5 mmol/L benzamidine, 1 mmol/L sodium orthovanadate, 10μg/ml aprotinin, 1 mmol/L PMSF, 10 μg/ml leupeptin, and 1 μg/ml pepstatin). The lysates were incubated with an anti Tie2 antibodies, immunocomplexes were recovered on Protein G-Sepharose and separated by SDS-PAGE, transferred to blotting membrane as described above and then probed with anti-Tie2, and with anti-phosphotyrosine antibodies 4G10 (Upstate Biotechnology, Inc, Lake Placid, NY). Monoclonal and polyclonal anti-Tek/Tie2 antibodies specific to the extracellular domain were a kind gift of Fu-Kuen Lin (Amgen, Thousand Oaks, CA). In experiments with pure proteins, rabbit polyclonal antibody against Tie2 (RG133, Regeneron Pharmaceutical) or commercially available polyclonal antibody against Tie2, (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) has been used.

New experiments demonstrate the preparation, cloning into cells, expression and activation effect on TIE2 of the natural Ang-1 and Ang-2, modified only to allow ease of identification and purification. In the below experiments, essentially similar techniques affect the plasmid, cell transfection, analysis of expression and secretion, and TIE2 activation were conducted with angiopoietin analogues as the preferred embodiments of the invention.

Example 2

A first Ang-1 chimera CJCC-Ang-1, which has a C-Jun coiled-coil

domain inserted upstream of the N-terminus of Ang-1, and a second Ang-1 chimera FBLD-Ang-1, which has a fibrogen-like domain inserted upstream of the N-terminus of Ang-1, were prepared by DNA recombinant techniques. Each was cloned separately into pFLAG vector. The chimeras and the resulting vector constructs are illustrated in Figure 8 of the accompanying drawings. They each include a Myc marker and a histidine tag, for identification, analysis and purification purposes, as described in Example 1. CJCC-Ang-1 is generally as illustrated in Figure 6 and FBLD-Ang-1 is generally as illustrated in Figure 2, of the accompanying drawings.

The vector plasmids contained in the chimeras, and Ang-1 as a control, were transfected into COS-1 cells as previously described and expressed and secreted into medium (both conditioned medium and cell extract). By Western blotting techniques, expression and secretion of Ang-1 and the CJCC-Ang-1 chimera were detected in CM (conditioned medium).

From the control experiment in which the Ang-1 containing vector was incorporated into the COS-1 cells, the secreted proteins were analysed from a purified sample, using SDS-PAGE analysis. Silver staining showed a band with a size of about 70 kD, corresponding to the size of Ang-1.

Partially purified Ang-1 was analysed by Western blotting, and revealed a major band with a size of 70 kD, as demonstrated on silver stained gel, and confirmed to be Ang-1.

Oligomerization patterns for Ang-1 and CJCC-Ang-1, purified as described above, were determined. They were both electrophoresed under non-reducing conditions and analysed by Western blotting. It appears that Ang-1 exists in the form of trimers and higher order of multimers, whereas CJCC-Ang-1 forms mainly dimers, trimers and higher order of multimers, with dimers being predominant over trimers.

Analysis of the expression of FBLD-Ang1 in COS-1 cells by Western blotting showed expression of the chimera which appeared as a monomer under reducing conditions, and as a polymer (mainly a trimer) under non-reducing conditions.

To determine TIE2 phosphorylation by Ang1 and the chimeras, HUVECs were serum starved and then treated with conditioned medium containing native Ang-1 or the chimeric constructs CCJC-Ang-1 or FBLD-Ang-1. Cell extracts were immunoprecipitated by anti-TIE-2 polyclonal antibody and then analysed by Western blotting using anti-phosphotyrosine antibody. The chimera FBLD-Ang-1 and the chimera CJCC-Ang-1 were found to be expressed and highly phosphorylated, apparently to a greater extent than Ang-1.

From this work it is concluded that Ang-1 and its chimeras CJCC-Ang-1 and FBLD-Ang-1 are expressed in COS-1 cells, and are suitable for expression in other appropriate cells and particularly in mammalian cells. Furthermore, according to their migration rates in SDS-PAGE gel, all of them were glycosylated. Ang-1 and FBLD-Ang1 exist in the form of trimers and higher order of multimers, rather than dimers not being seen in these two products. However, CJCC-Ang-1 forms dimers, trimers and higher order of multimers, dimers being predominant over trimers. Ang-1 was partially purified with only one major contaminating band seen in silver stained SDS-PAGE gel. It is shown that TIE2 can be phosphorylated by Ang1 and its chimeras. Thus there is disclosed both a method for preparing angiopoietin analogs comprising at least two angiopoietin binding domains capable of binding to and activating receptors involving angiogenesis, and such analogues themselves.

It will be understood that modifications and variations to the products and processes for their preparation and use can be made, based upon the disclosure and teachings contained herein. Such modifications are to be regarded as within the scope of the present invention. The scope thereof is not to be

construed as limited to the precise embodiments described herein.

REFERENCES

[1] Suri et al., "Requisite Role of Angiopoietin-1, a Ligand for the TIE2 Receptor, during Embryonic Angiogenesis", Cell; Vol. 87, 1171-1180, December 27, 1996.

[2] Davis et al., "Isolation of Angiopoietin-1, a Ligand for the TIE2 Receptor, by Secretion-Trap Expression Cloning", Cell; vol. 87, 1161-1169, December 27, 1996.

WHAT I CLAIM IS:

1. Angiopoietin analogs comprising proteinaceous molecules having at least two angiopoietin-like binding domains capable of binding to and activating receptors involved in angiogenesis, by associating pluralities of said receptors.

2. Angiopoietin analogs according to claim 1, wherein said angiopoietin-like binding domains are TIE-2 binding domains which bind to TIE-2 receptors expressed on membranes of endothelial and early hematopoietic mammalian cells.

3. Angiopoietin analogs according to claim 2 wherein the TIE-2 binding domains are amino acid sequences having substantial homology to the fibrinogen-like domain of naturally occurring angiopoietin-1 so as to comprise TIE-2 binding domains, said TIE-2 binding domains being separated by a biologically acceptable spacer domain.

4. Angiopoietin analogs according to claim 3 wherein the spacer domain is an amino acid sequence having substantial homology to the coiled-coil domain of naturally occurring angiopoietin-1.

5. Angiopoietin analogs according to claim 3 wherein the spacer domain is a non-proteinaceous synthetic linker sequence of biochemically acceptable chemical constitution.

6. Angiopoietin analogs according to claim 3 or claim 5 wherein the linker sequence is a polysaccharide sequence derived from hydroxyethyl starch or dextran.

7. Angiopoietin analogs according to claim 3 or claim 5 wherein the linker sequence is polyethylene glycol.

8. An angiopoietin-vascular endothelial growth factor analog comprising a proteinaceous molecule having at least one angiopoietin-like TIE2 binding domain and at least one VEGF-like tyrosine kinase binding domain, chemically linked together, optionally through the intermediary of a biochemically acceptable spacer or linker sequence.

9. An angiopoietin-vascular endothelial growth factor analog according to claim 8 wherein the Tie-2 binding domain is an amino acid sequence having substantial homology with the fibrinogen domain of naturally occurring angiopoietin-1.

10. Angiopoietin analogs according to claim 3 and further including at least two terminal KDR binding domains.

11. Angiopoietin analogs according to claim 3, claim 5, claim 8 or claim 9 wherein the spacer domain is a leucine zipper.

12. Angiopoietin analogs according to claim 11 wherein the leucine zipper is substantially homologous to that of c-jun.

13. Angiopoietin analogs comprising proteinaceous molecules having a first terminal amino acid sequence exhibiting substantial homology to the fibrinogen-like domain of naturally occurring angiopoietin-1, an intermediate amino acid sequence exhibiting substantial homology to the coiled-coil domain of naturally occurring angiopoietin-1, and a second terminal oligomerization sequence.

14. Angiopoietin analogs according to claim 10 wherein the spacer domain is a leucine zipper.

15. Angiopoietin analogs according to claim 14 wherein the leucine

zipper is substantially homologous to that of c-jun.

16. Genetic elements including polynucleotide sequences coding for and capable of expressing proteinaceous molecules according to any of claims 1 - 15.

17. Genetically engineered mammalian cells containing genetic elements according to claim 16.

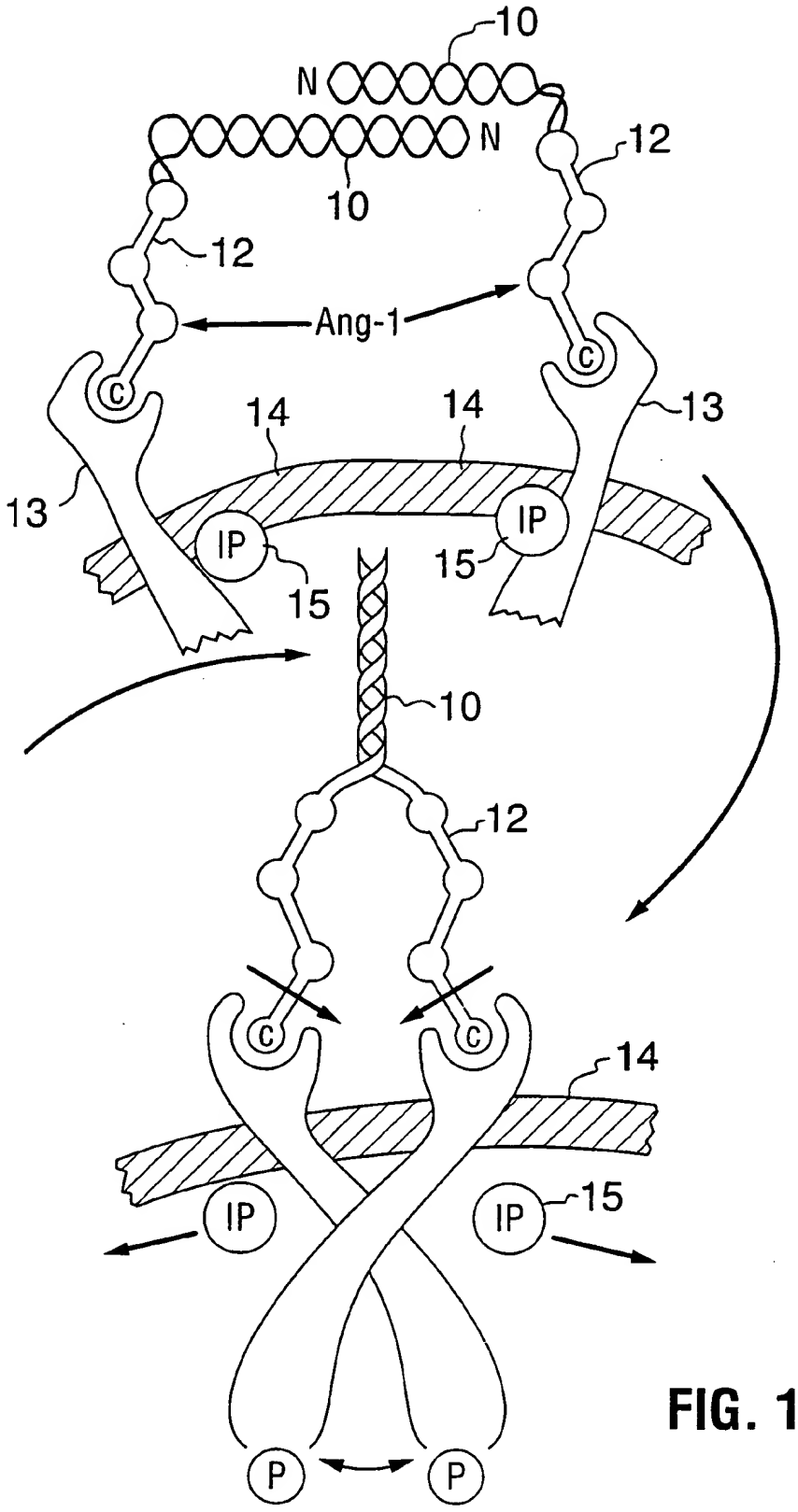

FIG. 1

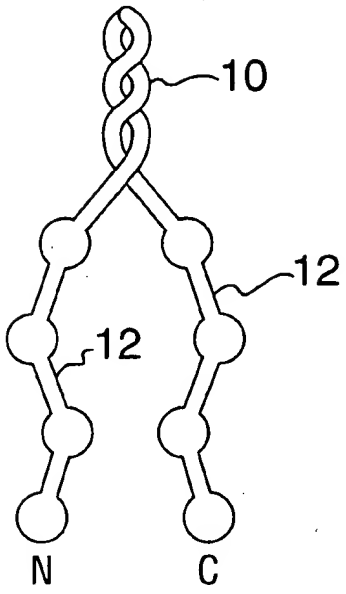

FIG. 2

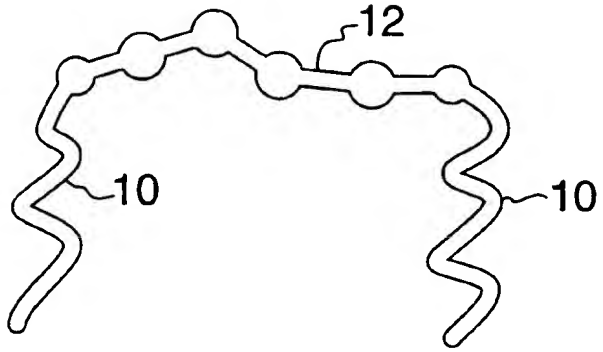

FIG. 3

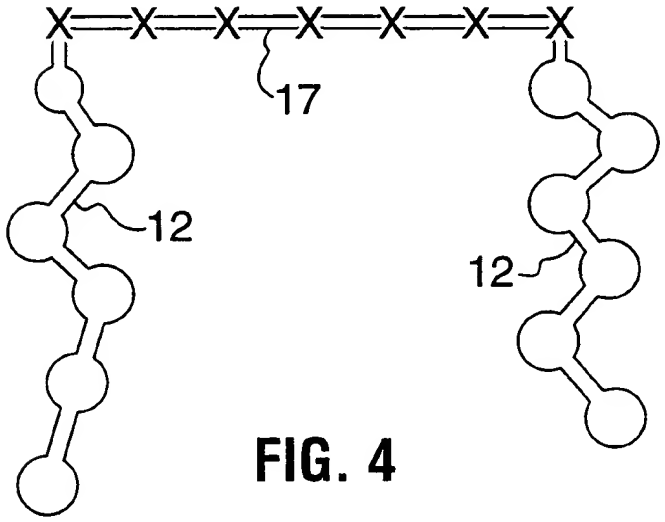

FIG. 4

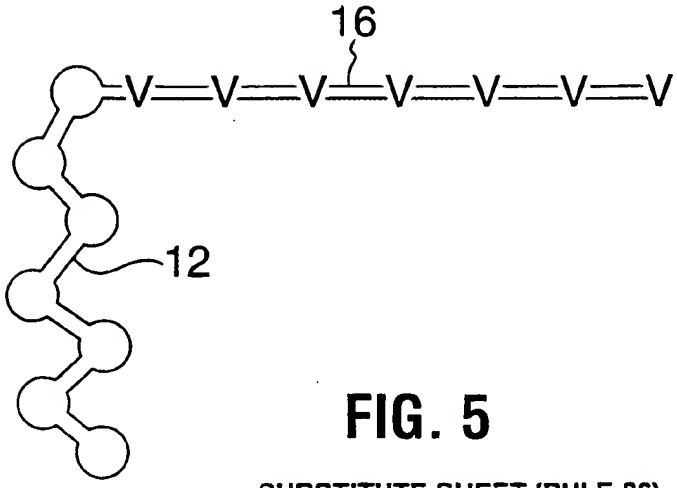

FIG. 5

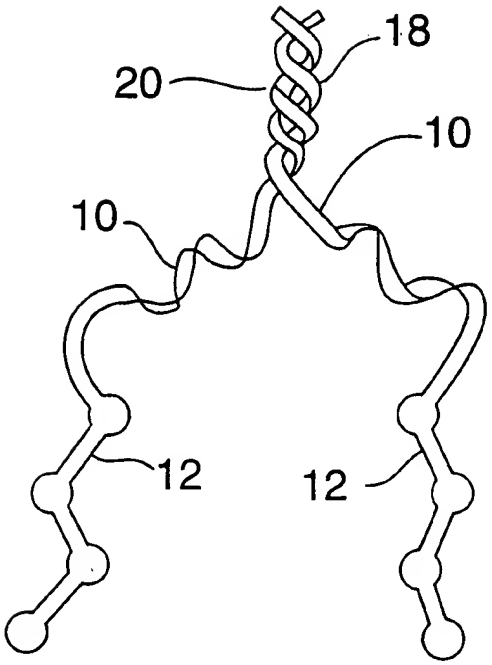

FIG. 6

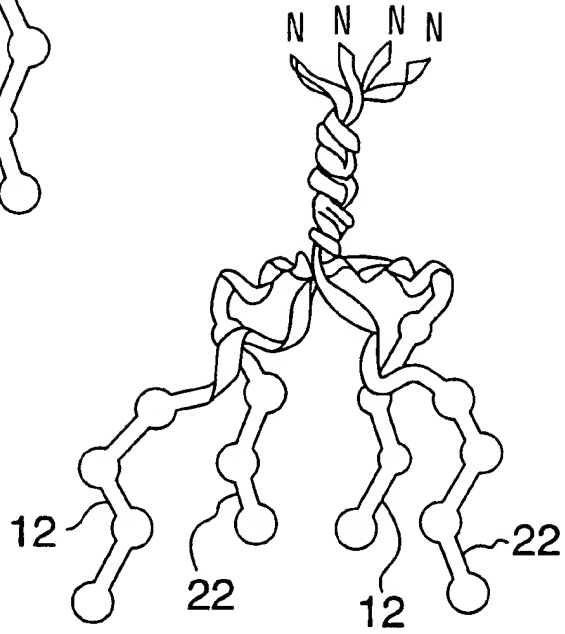

FIG. 7

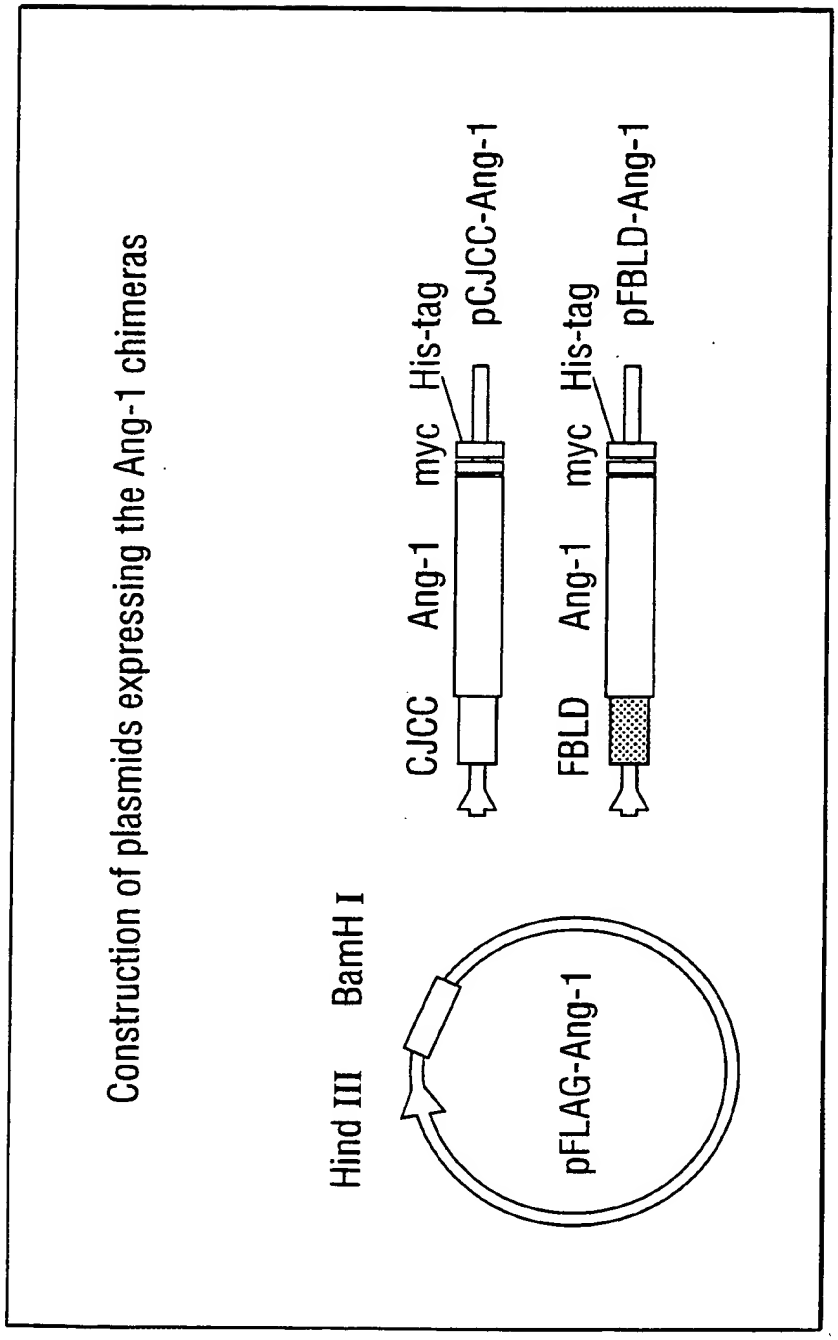
Construction of plasmids expressing the Ang-1 chimeras
Hind III
BamH I
pFLAG-Ang-1
CJCC
Ang-1
myc
His-tag
pCJCC-Ang-1
FBLD
Ang-1
myc
His-tag
pFBLD-Ang-1

FIG. 8